Antibody response to the 45 kDa *Candida albicans* antigen in an animal model and potential role of the antigen in adherence

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The *Candida* antigen CR3-RP (complement receptor 3-related protein) is supposed to be a 'mimicry' protein because of its ability to bind antibody directed against the α subunit of the mammalian CR3 (CD11b/CD18). This study aimed to (i) investigate the specific humoral isotypic response to immunization with CR3-RP *in vivo* in a rabbit animal model, and (ii) determine the role of CR3-RP in the adherence of *Candida albicans in vitro* using the model systems of buccal epithelial cells (BECs) and biofilm formation. The synthetic *C. albicans* peptide DINGGGATLPQ corresponding to 11 amino-acids of the CR3-RP sequence DINGGGATLPQALXQITGVIT, determined by N-terminal sequencing, was used for immunization of rabbits to obtain polyclonal anti-CR3-PR serum and for subsequent characterization of the humoral isotypic response of rabbits. A significant increase of IgG, IgA and IgM anti-CR3-RP specific antibodies was observed after the third ($P<0.01$) and the fourth ($P<0.001$) immunization doses. The elevation of IgA levels suggested peptide immunomodulation of the IgA1 subclass, presumably in coincidence with *Candida* epithelial adherence. Blocking CR3-RP with polyclonal anti-CR3-RP serum reduced the ability of *Candida* to adhere to BECs, in comparison with the control, by up to 35 % ($P<0.001$), and reduced biofilm formation by 28 % ($P<0.001$), including changes in biofilm thickness and integrity detected by confocal laser scanning microscopy. These properties of CR3-RP suggest that it has potential for future vaccine development.

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INTRODUCTION

Candida albicans is the most frequently isolated fungal pathogen associated with infection of immunocompromised patients in hospital settings. The high propensity to cause disease is due to the expression of many virulence factors including highly immunogenic cell surface proteins (Alberti-Segui *et al.*, 2004; Jeng *et al.*, 2005; Pietrella *et al.*, 2006) able to trigger cellular and humoral response (Viudes *et al.*, 2001; López-Ribot et al., 2004; Omaetxebarria *et al.*, 2005; Fukuizumi *et al.*, 2006). Additionally, a variety of cell surface molecules expressed by *Candida* help it to evade the host immune response by mimicry of host receptors (Gustafson *et al.*, 1991; Phan *et al.*, 2007). The receptor MAC-1 (CD11b/CD18) on lymphocytes is the surface β_2 integrin that mediates lymphocyte adhesion to *C. albicans* (Forsyth & Mathews, 2002). However, *Candida* is not only the target for MAC-1, but also CR3-RP identified on the yeast surface might be classified as a member of the integrin family due to its antigenic, structural and functional relation to the α subunit of the mammalian neutrophil receptor CD11b/CD18 (Gilmore *et al.*, 1988;

Abbreviations: BEC, buccal epithelial cell; CLSM, confocal laser scanning microscopy; RT, room temperature; XTT, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide.

The GenBank/EMBL/DDBJ accession number for the N-terminal sequence fragment of CR3-RP is P85437.

Hostetter *et al.*, 1990; Hostetter, 1996). Binding the human complement fragment iC3b to this receptor results in inhibition of opsonization and partial blocking of phagocytosis (Hostetter *et al.*, 1984; Heidenreich & Dierich, 1985). Several putative fragments of CR3-RP have been reported: these include a 165 kDa fragment (Hostetter *et al.*, 1990), 66, 55 and 42 kDa fragments (Alaei *et al.*, 1993), and 42 and 32 kDa fragments (Bujdáková *et al.*, 1999). Furthermore, a gene encoding a 188 kDa integrin with 18 % homology to the human CR3 has also been described in *C. albicans* (Gale *et al.*, 1996). Recently, the effect of oestrogen on CR3-RP expression was studied (Warolin *et al.*, 2005). Others reported that the transcription of CR3-RP was found to be dependent on Ca^{2+} ions and the yeast–mycelial transformation; its expression being higher in germ tubes and in the mycelial form (Spötl *et al.*, 1994; Bujdáková *et al.*, 1997).

C. albicans is able to bind to collagen, laminin or fibronectin via special surface molecules (Makihira *et al.*, 2002; Cateau *et al.*, 2007), and CR3-RP has been reported to participate in adhesion to endothelial cells (Würzner *et al.*, 1996). Adhesion of *C. albicans* to host cells is a crucial step in establishment of candidiasis. Moreover, it is also critical for an early phase in biofilm formation, the phenomenon associated with the new classes of diseases correlated with using medical devices (Kojic & Darouiche, 2004). Several genes that code for proteins that enhance the adherence capacity of *C. albicans* have been reported (Ibrahim *et al.*, 2005; Nailis *et al.*, 2006; Nobile *et al.*, 2006). However, anti-*Candida* antibodies can reduce *Candida* binding to different surfaces (Rodier *et al.*, 2003; Elguezabal *et al.*, 2004; López-Ribot *et al.*, 2004). To that end, the aim of this study was to (i) investigate the specific humoral isotypic response to immunization with CR3-RP *in vivo* in a rabbit animal model, and (ii) determine the role of CR3-RP in adherence of *C. albicans in vitro* using the model systems of buccal epithelial cells (BECs) and biofilm formation.

METHODS

Purification and analysis of the 45 kDa protein. The strain *C. albicans* K2 (CCY 29-3-162 from the Culture Collection of Yeasts (CCY), Institute of Chemistry, Slovak Academy of Sciences), originally isolated from vaginal candidiasis, was selected based on the high expression of CR3-RP (Bujdáková *et al.*, 1997). Total protein extract (100 ml) was obtained from 6 l germ tube suspension (10^7 germ tubes ml^{-1}) by breakage with glass beads (diameter 2 mm; Sigma-Aldrich) in breaking buffer (20 %, v/v, glycerol; 0.1 M Tris/HCl, pH 8.0; 1 mM DTT; 1 mM PMSF; all from Sigma-Aldrich). Germ-tube formation was induced as described previously (Bujdáková *et al.*, 1997). Briefly, an overnight yeast culture of *C. albicans* grown in yeast peptone dextrose (YPD) medium was washed three times with 0.85 % NaCl, then centrifuged (5000 ***g***, 10 min) and resuspended in 0.85 % NaCl in a new Falcon tube. This culture was ready for the starvation phase, which was performed at 28 °C for 2 h with gentle shaking (120 r.p.m.). Afterwards, the cell suspension was centrifuged again and adjusted to 10^7 cells ml^{-1} in RPMI medium with the addition of 300 mg *N*-acetylglucosamine l^{-1}, and adjusted with 50 mM Na_2HPO_4/KH_2PO_4 (all from Applichem) to pH 7.0. After 2 h incubation at 37 °C with shaking (180 r.p.m.), almost 95 % of the *C. albicans* in yeast form was transformed to germ tube form. The germ tubes were collected by centrifugation (5000 ***g***, 10 min) and the sediment was resuspended in 40 ml breaking buffer. Then the suspension was divided into 1 ml aliquots in 2 ml Eppendorf tubes and an approximately equal volume of glass beads was added to every tube. Cells were disrupted by vortexing 40 times at high speed for 1 min. Between vortexing steps, the tubes were cooled in an ice bath for 15 s. Then broken cells were centrifuged again (10 000 g, 4 °C, 10 min) and the supernatant containing protein extract was collected and dialysed in 20 mM Tris/HCl (pH 7.5) overnight. Then the extract was transferred into a new Falcon tube and 1 mM PMSF was added. An aliquot (4 ml) of the protein extract was stored at −20 °C until needed and the remaining sample was concentrated by filtration through an Amicon device (Millipore) to 4 ml. Partial purification of the 45 kDa protein (Bujdáková *et al.*, 1999) from the protein extract concentrate was performed by FPLC with Superose 12 P (Amersham) column (2 × 40 cm) equilibrated with 0.02 M NH_4HCO_3. Fractions were separated by SDS-PAGE in a 12.5 % polyacrylamide gel (Applichem), and the ~45 kDa band was extracted and subjected to FPLC again. Purified samples were concentrated by a Microcon YM 10 filter (Millipore) to 100 µl volume, and analysed by SDS-PAGE, followed by blotting to both nitrocellulose and PVDF membranes (both from Serva). The reaction of the protein with monoclonal antibody OKM1 (American Type Culture Collection cultures CRL-8026, recognizing the α chain of human CR3, CD11b) on nitrocellulose membrane was performed as described previously (Bujdáková *et al.*, 1999). Briefly, after overnight blocking at 4 °C in Tris buffered saline (TBS) buffer (50 mM Tris/HCl, pH 7.4; 150 mM NaCl) supplemented with 5 % (w/v) non-fat dry milk, the membrane was incubated with the OKM 1 antibody diluted 1 : 40 in TBS for 1 h at room temperature (RT). After three washing steps in TBS, 0.05 % (v/v) Tween 20 (Merck) (TBST), anti-rabbit IgG conjugated with alkaline phosphatase (Sigma-Aldrich) diluted 1 : 30 000 in TBS was added. The membrane was incubated for 1 h at RT. After four washes in TBST, the membrane was developed using Sigma Fast BCIP/NBT tablet (Sigma-Aldrich) dissolved in 10 ml deionized water. The colour reaction was stopped with deionized water.

The PVDF membrane was stained with amido black solution (Applichem) and subjected to N-terminal sequencing in two laboratories independently: at Innsbruck Medical University, where 11 amino acids were sequenced, and at the Institute of Organic Chemistry and Biochemistry, Academy of Sciences of the Czech Republic, where 21 amino acids were identified by Edman degradation on an ABI 491 protein sequencer.

Preparation of polyclonal antibody to CR3-RP, ELISA and Western blotting. A synthetic peptide with >90 % purity, as revealed by HPLC and MS analysis, was generated by KJ Ross-Petersen, Denmark. Its sequence corresponded to the first 11 amino acid residues (DINGGGATLPQ) at the N-terminus of the CR3-RP protein. This peptide was used to raise polyclonal anti-CR3-RP serum by immunization of rabbits (female, 8-weeks-old, 2250 g weight, variety Hyla; Research Institute of Animal Production, Nitra, Slovakia). The rabbits were split in two groups of three each. Pre-immune serum samples were collected as a negative control before the experiment. The two groups were immunized by: (i) an intramuscular injection of 40 µg CR3-RP mixed with complete Freund's adjuvant (Sigma-Aldrich) at day 0, boosted with another four doses in incomplete Freund's adjuvant (80 µg, 160 µg, 300 µg, 300 µg) 2 weeks apart (five immunizations in total); or (ii) heat inactivated *Candida* yeast-form cell suspension in sterile saline (10^7 ml^{-1}) administered intravenously in a marginal ear vein three times, 2 weeks apart. Both groups of rabbits were bled 2 weeks after the last injection. Antibody titres were determined by ELISA in 96-well plates

(Sarstedt) (Voller, 1978). The wells were coated with 100 µl (per well) CR3-RP diluted in PBS at final concentration 5 µg ml^{-1}, supplemented with 100 µl coating buffer (0.1 M $NaHCO_3$, pH 9.6), and incubated overnight at 4 °C. After one washing step, non-specific binding was blocked by 1 h incubation in 3 % (w/v) non-fat dry milk (Merck) in PBS, 0.05 % (v/v) Tween 20 (Sigma-Aldrich) (PBST). The peptide was incubated with several dilutions of the anti-CR3-RP serum. After three washing steps with PBST, goat anti-rabbit IgG conjugated with alkaline phosphatase (Sigma-Aldrich) diluted 1 : 30 000 was added and the plate was incubated for 1 h at RT. After four additional washing steps, alkaline phosphatase substrate containing *p*-nitrophenylphosphate (Sigma-Aldrich) was used for developing the results. The reaction was stopped with 3 M NaOH and the absorbance measured at 490 nm using a microplate reader (MRX; Dynex). For immunoblotting, 15 µl *C. albicans* K2 protein extract was separated by 12.5 % SDS-PAGE and transferred to nitrocellulose membrane (Serva). After overnight blocking at 4 °C in TBS buffer supplemented with 5 % (w/v) non-fat dry milk, the membrane was incubated with the polyclonal anti-CR3-RP serum (diluted 1 : 100 in TBS) for 1 h at RT. After three washing steps in TBST, anti-rabbit IgG conjugated with alkaline phosphatase (Sigma-Aldrich) diluted 1 : 30 000 in TBS was added. The membrane was incubated for 1 h at RT. After four washes in TBST, the membrane was developed using Sigma*Fast* BCIP/NBT tablets (Sigma-Aldrich) dissolved in deionized water (10 ml per tablet). The colour reaction was stopped with deionized water.

Production of CR3-RP specific IgG, IgM and IgA antibodies in a rabbit model, and ELISA. The CR3-RP specific rabbit IgG, IgM and IgA isotype antibodies induced by immunization with either CR3-RP in Freund's adjuvant or whole *Candida* cells were detected using a modified ELISA. Briefly, 10 µg CR3-RP in carbonate/bicarbonate buffer pH 9.6 (100 µl per well) was applied onto Immulon 4 HBX microplates (Dynex) for 18 h at 4 °C, and the plates were coated with 2 % (w/v) non-fat milk (KPL) in PBST pH 7.2. Sequential titrations of rabbit antisera were performed stepwise with the pre-coated CR3-RP overnight at 4 °C. Immune complexes were tested for alkaline phosphatase-labelled protein A (Calbiochem) for IgG, and with alkaline phosphatase-labelled goat anti-rabbit IgA and IgM (Bethyl Laboratories) for antisera IgA and IgM levels, respectively. The enzyme reaction was developed with the BluePhos microwell phosphatase substrate system (KPL) and absorbance measured at 630 nm (MRX II; Dynex). The results were expressed as arbitrary units, defined as the amount of the anti-CR3-RP specific IgG, IgA and IgM present in 1 : 1000 dilution of the reference whole cell antiserum, and were calculated as a percentage of the reference whole cell antiserum (100 %) and presented as the mean ± SEM of three rabbits per group.

Computational and statistical analysis. Results from *in vitro* experiments were calculated as mean values ± SEM. Similarly, *in vivo* results were expressed as the mean ± SEM for each examined group. Normality of distribution was evaluated according to the Shapiro–Wilk test at the 0.05 level of significance. Statistical comparison between groups was performed using one-way analysis of variance (ANOVA) and post-hoc Bonferroni and Tukey tests. The results were significant if the difference between the analysed groups equalled or exceeded the 95 % confidence level ($P<0.05$). Analyses were performed using Origin 7.5 Pro software (OriginLab).

Adherence to BECs. Adherence of *Candida* cells, with and without pre-incubation with the polyclonal anti-CR3-RP serum, to BECs was performed according to Moragues *et al.* (2003) with some modifications. Briefly, human BECs were collected from six healthy volunteers by gently scraping cheek mucosa with sterile wooden spatulas. After centrifugation (1000 ***g***, 10 min, RT), the cells were washed three times with PBS and suspended at 10^5 cells ml^{-1} in PBS. *C. albicans* cells from an overnight culture in YPD were washed with PBS three times and diluted in PBS to a density of 10^8 cells ml^{-1}. For testing the polyclonal anti-CR3-RP serum, 100 µl *Candida* suspension was harvested by centrifugation at 2000 ***g*** for 5 min at 4 °C, and washed gently three times with 500 µl blocking solution (3 % gelatin in PBS). Then, 50 µl polyclonal anti-CR3-RP serum diluted 1 : 100 in PBS was mixed with *Candida* cells and incubated on ice for 1 h. This suspension was washed three times with PBST, collected and used directly for incubation with BECs. In the control experiment, *Candida* cells were treated only with PBS instead of polyclonal anti-CR3-RP serum. The 500 µl BEC suspension was mixed with 50 µl *Candida* suspension treated with polyclonal anti-CR3-RP serum or PBS and incubated for 2 h with gentle agitation (100 r.p.m.) at 37 °C. The mixtures were centrifuged (1000 ***g***, 10 min, RT), the supernatants discarded and the pellets resuspended in 50 µl PBS by gentle rotary mixing. The cells were then counted with a haemocytometer. A minimum of 100 BECs were analyzed using light microscopy at 400 × magnification to determine the number of adherent *Candida* cells. The number of adherent yeast upon pretreatment with specific antibody and pre-immune serum was calculated as a percentage of those pretreated with PBS in the parallel control experiment (100 %). The final percentage of adherent cells was determined as the difference between the percentage of *Candida* cells treated with polyclonal anti-CR3 serum and those treated with pre-immune serum. For capturing an image, the suspension of BECs with *Candida* cells was dropped onto a glass slide, dried at RT, and then fixed with methanol for 10 s, covered with 5 µl crystal violet and dried again at RT (Fig. 1).

Determination of cell surface hydrophobicity (CSH) and biofilm formation. Yeast *C. albicans* K2 used for both hydrophobicity and biofilm assays was cultivated on Sabouraud dextrose agar (Biomark Laboratories) at 28 °C for 24 h. A large loop of cells was transferred into yeast nitrogen base with amino acids (YNB) broth (Sigma-Aldrich) supplemented with 0.9 % D-glucose (Applichem), and after overnight incubation at 37 °C, the cells were centrifuged at 4000 ***g*** for 5 min and washed twice with 0.5 ml PBS. The cells were resuspended in 1.5 ml YNB broth and the suspension was adjusted to OD_{600} 1.0. CSH was measured according to the method of Klotz *et al.* (1985) using the biphasic separation method with *n*-octan (Merck) overlay.

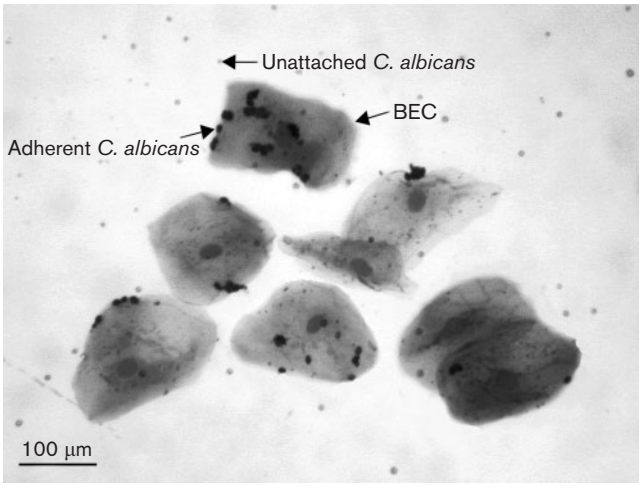

Fig. 1. Binding of *C. albicans* yeast cells to human BECs visualized by crystal violet staining. Arrows indicate adherent, as well as non-adherent *C. albicans* and BECs.

The relative percentage of CSH was calculated as follows: [(OD_{600} of the control − OD_{600} after octane overlay)/OD_{600} of the control] × 100.

Biofilm was formed using polystyrene 96-well plates (Sarstedt) in 100 µl YNB medium supplemented with 0.9 % D-glucose at 37 °C and quantified by its ability to reduce XTT [2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-2H-tetrazolium-5-carboxanilide] sodium salt to water soluble formazan (Sigma-Aldrich) as described by Li *et al.* (2003). Parallel experiments were performed with pre-incubation of *C. albicans* cells with polyclonal anti-CR3-RP serum and pre-immune serum. The yeast suspension prepared as described above, was centrifuged, the cell pellet treated by three washes in 3 % (w/v) gelatin (Oxoid) dissolved in PBS, and then incubated with 50 µl polyclonal anti-CR3-RP serum or pre-immune serum, both diluted 1 : 100 in PBS, for 1 h on ice. Serum was removed by centrifugation and three washes in PBST and the yeast cells were used for the hydrophobicity and biofilm assays.

Confocal laser scanning microscopy (CLSM). Mature biofilms formed on polyethylene coverslips in Petri dishes (both from Sarstedt), prepared according to Li *et al.* (2003), were transferred to new Petri dishes and 20 µl tetramethyl rhodamine methyl ester perchlorate (TMRM; excitation wavelength 549 nm, emission wavelength 573 nm; Invitrogen), diluted in distilled deionized water to final concentration of 5 µM, was applied to each coverslip. Stained biofilms were observed with a LSM 510 META confocal laser scanning microscope head, mounted on an Axiovert 200 M inverted microscope (both from Carl Zeiss, Germany). In fluorescence mode, the 543 nm line of the He–Ne laser excited the sample and a 545 nm dichroic mirror with 565–615 nm band-pass emission filter were used for fluorescence detection. In transmission mode the laser light at 543 nm passing through the sample was detected by a photodiode attached to the microscope condenser. A 20 × 0.75 PlanApochromat dry objective was used, with the confocal pinhole opening corresponding to 1 Airy unit. The half-width of the estimated point-spread function in Z direction was 1.8–2 µm (in the 500–600 nm wavelength range), therefore we used 0.9 µm *Z*-axis sampling for three-dimensional (3D) data recording. For each image, 153 × 153 µm area was scanned with a resolution of 512 × 512 pixels, 16 × line averaging and 8-bit quantization. The images were further processed with LSM Image Examiner software. Biofilm images were either displayed individually as two-dimensional plots, or reconstructed in 3-D projections. Vertical (*xz*) sections or side views of the 3D reconstructed images were used to determine biofilm thickness and architecture. The thickness was estimated from the outer edges of the area, where TMRM signal gain intensity was above half of its maximum.

RESULTS AND DISCUSSION

Purification of CR3-RP, and preparation and evaluation of polyclonal anti-CR3-RP serum

The human CR3, also known as MAC-1 (CD11b/CD18), expressed on leukocytes is a principal adhesion receptor for binding iC3b-opsonized pathogens. The existence of integrin-like proteins in *C. albicans* with affinity for complement conversion product iC3b has been suggested by several authors (Eigentler *et al.*, 1989; Hostetter *et al.*, 1990; Gustafson *et al.*, 1991; Alaei *et al.*, 1993; Spötl *et al.*, 1994; Hostetter, 1996; Bujdáková *et al.*, 1999); however, information about its contribution to *Candida* virulence is very limited. *C. albicans* expressing CR3-RP was able to form rosettes consisting of iC3b-coated sheep erythrocytes bound to germ tubes or mycelial forms. Furthermore, immunofluorescence, as well as ELISA results, revealed CR3-RP cross-reactivity with anti-human CR3 mAb named OKM1 (Eigentler *et al.*, 1989; Bujdáková *et al.*, 1997, 1999), which is able to block the function of the lectin domain of CD11b. CR3-RP was purified as described in Methods and the molecular mass was estimated to be around 45 kDa (Fig. 2). N-terminal sequencing of the putative CR3-RP protein revealed two amino acid motifs with sequences DINGGGATLPQ and DINGGGATLPQALXQITGVIT (the latter sequence was submitted to the UniProt Knowledgebase, under UniProt accession number P85437, entitled CR3-RP in *C. albicans*). The first 11 amino acids exhibited 100 % homology between the protein samples sequenced independently in two different laboratories. This identical sequence was used to search the Swiss-Prot protein database using BLAST available online, but revealed no homology to any *Candida* proteins or other fungal proteins already published. The sequence corresponding to the first 11 amino acids was synthesized and this peptide was used for immunization of rabbits as described in Methods.

Immunization of rabbits with CR3-RP and characterization of the humoral response

We hypothesized that CR3-RP, as further characterized in this study, belongs to the group of surface molecules adopted by pathogenic micro-organisms to improve entry into the host (Sturtevant & Calderone, 1997; Taborda & Casadevall, 2002; Nosanchuk *et al.*, 2003). These antigens are usually mannoproteins able to elicit a response from the host immune system. Successful anti-*Candida* defence requires tight cooperation of the different mechanisms of innate and adaptive immune responses (Roeder *et al.*, 2004; Netea *et al.*, 2006). In spite of fact that cell-mediated

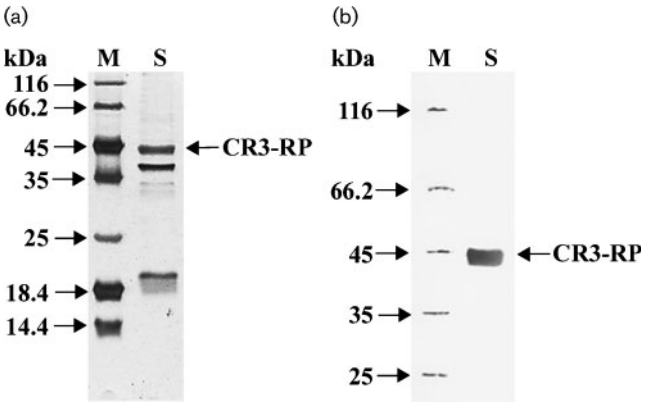

Fig. 2. (a) SDS-PAGE separation of *Candida* protein extract after liquid chromatography visualized by silver staining assay. (b) The interaction of the CR3-RP blotted to nitrocellulose membrane with polyclonal anti-CR3-RP serum visualized by alkaline phosphatase assay. M, Protein size marker; S, sample.

immunity is important during the first contact with fungal pathogen, the contribution of the specific humoral response has also been demonstrated in recent years (López-Ribot *et al.*, 2004; Cutler, 2005; Paulovičová *et al.*, 2005). The induction of an antigen-specific humoral response was evaluated by IgG, IgM and IgA anti-CR3-RP specific sera levels in immunized rabbits (Fig. 3). While IgM is the first antibody produced in the humoral response, mediating opsonization of micro-organisms by activation of the classical complement pathway, the IgG class has a protective role, including reduction of the *C. albicans* binding capacity to host and medical devices (Kondori *et al.*, 2003; Rodier *et al.*, 2003), and serum IgA, mainly its subclass unit antibody, is an important part of mucosal immunity initiating numerous host defence activities, such as phagocytosis, cytotoxicity and respiratory burst (Morton *et al.*, 1996; van Spriel *et al.*, 2002). The experimental sera were evaluated by ELISA and the isotypic distribution induced by CR3-RP alone and/or heat-inactivated whole *Candida* cells was determined. The prime–boost immunization strategy enhanced, in particular, polyclonal production of CR3-RP–specific IgG, IgA and IgM isotype antibodies. CR3-RP administration induced the highest increase of anti-CR3-RP IgG and IgA levels after the third ($P<0.01$) and later ($P<0.001$) immunization doses. Comparison of induced specific anti-CR3-RP-isotypes with the results from whole *Candida* cells, especially after the second booster, demonstrated a higher immunogenicity of CR3-RP. While induction of IgM and IgG antibodies has been described for many *Candida* antigens (Rodier *et al.*, 2003; Torosantucci *et al.*, 2005), the marked elevation of IgA levels in serum suggested CR3-PR peptide immunomodulation of the IgA1 subclass, presumably in coincidence with *Candida* epithelial adherence.

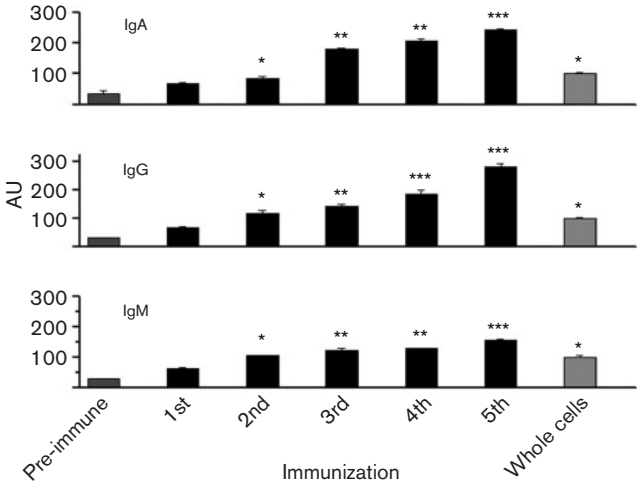

Fig. 3. Specific humoral immune response induced by CR3-RP immunization. The experimental data are expressed as geometric means ± SEM. The normality of the distribution of each data group was evaluated at a 0.05 level of significance. The results are expressed as arbitrary units (AU), which are defined as the amount of anti-CR3-RP specific IgG, IgA and IgM present in 1 : 1000 dilution of the reference whole cell antiserum, calculated as a percentage of reference whole cell antiserum (100 %). Comparisons of all examined groups with pre-immune baseline results were performed by ANOVA. Levels of significance: ***, $0.000<P<0.001$; **, $0.001<P<0.01$, *, $0.01<P<0.05$. Differences were considered significant where $0.01<P<0.05$.

Role of CR3-RP in adherence to BECs and biofilm formation, determination of CSH, and CLSM

To test the hypothesis about participation of CR3-RP in adherence, we coated fungal adherence receptors with an antibody that can reduce the adherence of *C. albicans* cells (Moragues et al., 2003; López-Ribot *et al.*, 2004). Upon treatment with the polyclonal anti-CR3-PR serum, the binding of *Candida* cells to BECs was reduced up to 35 % in comparison with the control sample ($P<0.001$). This suggested that the polyclonal anti-CRP-PR serum might also affect biofilm formation – a complex process in which adherence is a determining factor (Blanco *et al.*, 2006; Blankenship & Mitchell, 2006). Indeed, we observed a reduction in biofilm formation, most likely due to a decreased adherence capacity mediated by blocking CR3-RP, by about 28 % in comparison with the control ($P<0.001$) (Fig. 4). As hydrophobicity of the yeast cell wall is an important factor affecting adherence to different substrates (Hazen *et al.*, 2001; Singleton *et al.*, 2005) and is directly correlated with the surface protein composition, we investigated how it is affected by CR3-RP. The percentage of hydrophobic cells decreased after the pre-incubation of *C. albicans* with polyclonal anti-CR3-RP serum from 20.1 to 9.5 % ($P<0.001$), suggesting that interaction of the CR3-RP with the antiserum affects the hydrophobicity status of the *Candida* population. Hence, a

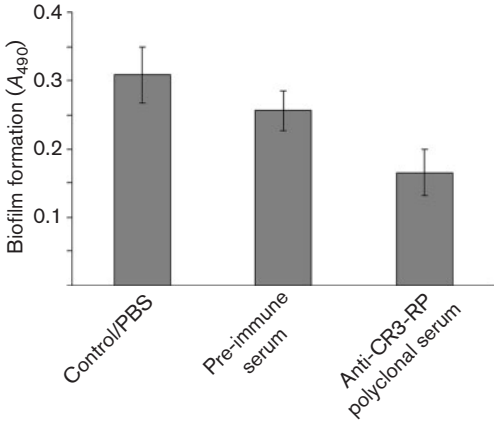

Fig. 4. Effect of pretreatment of *C. albicans* cells with polyclonal anti-CR3-RP serum on biofilm formation. The biofilm was quantified by XTT reduction assay as described in Methods. The experimental data are expressed as geometric means ± SEM.

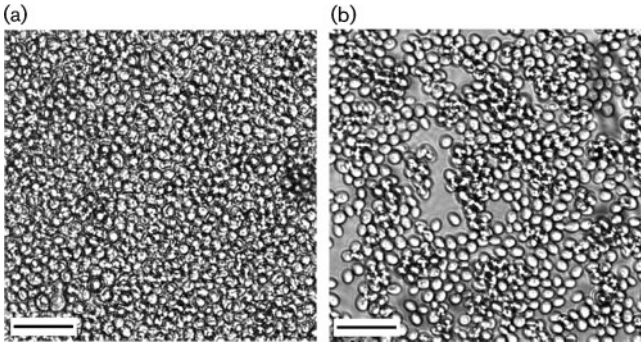

Fig. 5. Biofilm images taken by CLSM in transmission mode. The biofilm was produced by *C. albicans* on polyethylene coverslips after 48 h cultivation without (a) and with (b) pre-incubation with polyclonal anti-CR3-RP serum. Bars, 20 μm.

change in the ratio of hydrophobic/hydrophilic cells in a *Candida* suspension can indirectly affect the capacity of *C. albicans* to adhere to BECs or to polystyrene microtitre plates in the biofilm model. Using CLSM, a corresponding reduction in biofilm thickness was observed, from 14.5 μm in the control to 9 μm in the sample pre-incubated with the polyclonal anti-CR3-RP serum ($P<0.05$). Moreover, a loss of biofilm integrity after pre-incubation with polyclonal anti-CR3-RP serum was observed (Fig. 5). Taken together our results provide evidence for the strong immunomodulatory efficiency of CR3-RP and its contribution to the adherence of *C. albicans*. These properties suggest that CR3-RP is a fungal antigen with a potential for vaccine development.

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